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ON THE UNIVALENT FRAGMENTS OF HUMAN 7 S GAMMA-GLOBULIN

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HYDROLYSIS of 7 *S* rabbit antibody by papain yields three 3·5 *S* fractions separable by chromatography on carboxymethyl cellulose[1,2]. Two of these fractions, designated I and II by Porter, resemble each other in possessing univalent antibody activity[3–5] and in antigenic characteristics and molecular weight. Fraction III lacks antibody activity. Palmer, Mandy and Nisonoff[6] recently demonstrated that fractions I and II are derived from different parent molecules rather than being fragments of the same molecule (as originally thought), and showed that the relative amounts of fractions I and II vary reciprocally, depending on the chromatographic behaviour of the parent γ-globulin. Confirmatory evidence for this concept was recently furnished by Stelos *et al.*, using another method[7].

Various methods of fractionating the papain digest of human 7 *S* γ-globulin have been developed[8–12]. Only the method of Franklin, however, is comparable with that of Porter in producing three fractions. These fractions, designated *A*, *C* and *B*, correspond to fractions I, II and III, respectively, of rabbit γ-globulin[9]. (A small fourth fraction, designated *D* and probably representing intact 7 *S* material, is sometimes observed.) According to Franklin's results, the relative amounts of *A* and *C* in digests of pooled human Cohn fraction II varied, depending on the chromatographic and electrophoretic behaviour of the parent γ-globulin, whereas the proportion of *B* was relatively constant[9]. The basic fraction was rich in the positively charged fragment *C* while the more rapidly migrating fraction contained primarily the more negatively charged fragment *A*. However, no conclusions were made on the basis of this observation on whether or not *A* and *C* are derived from the same parent molecule. The apparent heterogeneity of Cohn fraction II in this respect might merely reflect its origin from γ-globulins of different individuals. The work recorded here attempted to clarify this point by sub-fractionating γ-globulin derived from single individuals on the basis of charge.

Sera from normal subjects, four patients with multiple myeloma, and three with an 'incomplete' anti-Rh antibody were the source of γ-globulin. In brief, the 7 *S* γ-globulins were isolated by diethylaminoethyl cellulose (DEAE)

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chromatography and separated into 'acidic' and 'basic' components by carboxymethyl cellulose chromatography or starch-block electrophoresis. These 7 *S* sub-fractions were then digested with papain and the resultant 3·5 *S* products were separated by sequential chromatography on carboxymethyl cellulose and DEAE columns. Experiments were also performed with fragments *A* and *C* of the 'incomplete' anti-Rh antibodies to evaluate their serological attributes.

Normal γ-globulin was prepared from 50-ml. samples of serum from two normal donors (P. S., Gm(a+), Inv(a+), and J. D., Gm(a-), Inv(a-)) by elution from a 4 × 40 cm DEAE column[13] with 0·01 M phosphate buffer, *p*H 8·0. This yielded pure 7 *S* γ-globulin as shown by immunoelectrophoresis[14]. A 250-ml. sample of each protein was further separated into four sub-fractions on a 2 cm × 30 cm carboxymethyl cellulose column, using stepwise elution as follows: buffer 1, 0·005 M phosphate, *p*H 6·6; buffer 2, 0·01 M phosphate, *p*H 6·6; buffer 3, 0·025 M phosphate, *p*H 6·9; buffer 4, 0·05 M phosphate, *p*H 7·1, plus 0·04 M sodium chloride. In a further experiment, normal γ-globulin was applied to a carboxymethyl cellulose column with 0·025 M phosphate, *p*H 6·9, plus 0·025 M sodium chloride to elute the 'acidic' and 'intermediate' portions; 0·05 M phosphate, *p*H 7·6, plus 0·5 M sodium chloride (buffer 5) was then used to elute the most 'basic' portion. The 7 *S* γ-paraproteins of two patients with multiple myeloma were prepared similarly by elution from a DEAE column. The γ-cryoglobulins of two other myeloma patients were isolated and purified by repeated cold precipitation and washing. All the globulin preparations were concentrated to 15 ml. by dialysis against polyethylene glycol and then against phosphate-buffered saline, *p*H 7·0. The solutions were made 0·01 M in cysteine and 0·002 M in disodium ethylenediamine tetraacetate; mercuripapain (Worthington Biochemicals) was then added in the amount of 1 per cent of the protein by weight. Digestion was allowed to proceed at 37° C for 16 h and was then stopped by dialysis against 100 ml. of 0·01 M phosphate buffer, *p*H 7·6, in the cold. The amount of dialysable material formed was determined by a modified Folin technique[15]. The digests were fractionated in the cold by the two-step chromatographic method of Franklin[9] (carboxymethyl cellulose columns at *p*H 7·6, followed by DEAE columns at *p*H 8·0). The same batch of ion exchanger and the same conditions of flow rate and buffer were used for each run. The amount of protein in each fraction was determined by optical density at 280 mμ. γ-Globulin possessing incomplete anti-D (anti-Rh_0) activity was isolated in the same manner as the γ-globulin devoid of demonstrable antibody activity for this specific antigen. Anti-Rh activity was assayed by standard methods[16]. In all negative serological tests,

serial dilutions were used to ensure that a prozone did not exist.

Table 1 shows the relative amounts of protein in the fractions (expressed as percentage of the parent protein) obtained from digests of normal γ-globulin (J. D.), its chromatographic sub-fractions, and the myeloma proteins. As shown, the most 'acidic' sub-fraction of normal γ-globulin (that is, the sub-fraction most easily eluted from the carboxymethyl cellulose column, and hence presumably possessing the lowest isoelectric point) yielded *A* but no *C*. The most 'basic' sub-fraction (that is, the sub-fraction eluted last) yielded mainly *C* and little *A*. The intermediate sub-fractions yielded intermediate ratios of *A* to *C*. Similar results were obtained with chromatographic sub-fractions of another normal γ-globulin (P. S.). The myeloma proteins resembled the artificially produced sub-fractions of normal γ-globulin in yielding markedly unequal amounts of *A* and *C*. (The property of cold precipitation was lost by the cryoglobulins after papain digestion, and was not present in any of the fractions or various mixtures thereof.) In confirmatory experiments, 'fast' and 'slow' sub-fractions of the two normal γ-globulins (P. S. and J. D.) were isolated by starch-block zone electrophoresis[17]. The relative amounts of *A* and *C* fragments obtained from papain digests of 'fast' and 'slow' sub-fractions isolated by starch-block electrophoresis were similar to those obtained from the 'acidic' and 'basic' chromatographic sub-fractions, respectively. Agar diffusion examination of the isolated *A*, *B* and *C* fragments of the 'acidic' portion obtained by chromatographic fractionation gave bands of identity with the comparable fragments obtained by isolation of the 'fast' γ-globulin from starch-block electrophoresis. The same was true of the papain fragments of the 'basic' (chromatographic) and 'slow' (starch-block) fractions of the 7 *S* γ-globulin.

Table 2 shows the representative results obtained with one (Ri) of three incomplete anti-Rh antibodies. The parent γ-globulin possessed incomplete anti-Rh activity as measured by antiglobulin test and by its ability to agglutinate *D*-positive erythrocytes treated with ficin or suspended in 30 per cent albumin. No agglutination was observed in saline. The papain digest of the Ri γ-globulin and its *A* and *C* fractions retained anti-Rh activity as measured by the antiglobulin test with rabbit anti-serum to the *A* and *C* fragments, but showed no detectable activity against red cells pre-treated with proteolytic enzymes (ficin, trypsin and papain) or suspended in albumin. The *B* fraction was inactive by all three methods. These results are in accord with those obtained by Fudenberg, Mandy and Nisonoff[18] with rabbit 7 *S* hæmagglutinins. (The localization of anti-globulin activity

Table 1. RELATIVE AMOUNTS OF FRACTIONS OBTAINED FROM PAPAIN DIGESTS OF VARIOUS GAMMA-GLOBULINS*

	Fraction						
	A	*C*	*A*+*C*	*B*	*D*	Peptides	Ratio *A*/*C*
Normal γ-globulin							
Total	34·7	27·9	62·6	28·6	0·8	8·0	1·24
Sub-fraction 1 ('acidic')	48·7	0	48·7	34·3	0	17·0	0
Sub-fraction 2	63·2	1·2	64·4	21·5	0	14·1	52·7
Sub-fraction 3	37·3	19·0	56·3	34·7	0·7	3·3	1·96
Sub-fraction 4	37·8	35·1	72·9	17·6	0	9·5	1·08
Sub-fraction 5 ('basic')	6·7	38·1	44·8	31·3	10·4	13·5	0·18
Paraproteins							
Myeloma 1	16·8	48·9	65·7	22·7	4·1	7·5	0·34
Myeloma 2	53·3	12·1	65·4	26·7	3·3	4·6	4·40
Myeloma 3 (cryoglobulin)	79·4	0·3	79·7	17·2	0	3·1	265·0
Myeloma 4 (cryoglobulin)	8·8	48·1	56·9	24·9	7·3	10·9	0·18

* All values expressed as percentages of the parent protein, except the ratio *A*/*C*.

Table 2. TITRES* OF ANTI-RH ANTIBODY (RI) AND ITS PAPAIN DIGEST FRACTIONS BY VARIOUS TECHNIQUES

γ-Globulin preparation	Method			
	Antiglobulin	Ficin†	Albumin	Saline
Intact (7 *S*)	1 : 10,000	1 : 10,000	1 : 200	—
Papain digest	1 : 1,250	—	—	—
Fraction *A*	1 : 1,000	—	—	—
Fraction *B*	—	—	—	—
Fraction *C*	1 : 1,000	—	—	—

* End-point of final dilution causing detectable reaction. Initial concentration of protein solutions was 10 mg/100 ml.

† Similar results were obtained when cells were treated with trypsin or papain.

of incomplete anti-Rh antibody to the *A* and *C* fragments was previously observed by Deutsch *et al.*[19].) In a further experiment, the intact anti-Rh γ-globulin (Ri) was sub-fractionated chromatographically into 'acidic' and 'basic' portions. The papain digest of the 'acidic' portion yielded mainly fraction *A* and little *C*, whereas the 'basic' portion yielded the reverse. As before, anti-Rh activity was confined to *A* and *C*. As a control on the chromatographic method, a part of the 'acidic' anti-Rh γ-globulin (*A*/*C* ratic 59 : 1) was mixed with a ten-fold excess of the 7 *S* γ-globulin (not sub-fractionated) of 'normal' sera lacking anti-Rh activity. The mixture of 'acidic' anti-Rh and of normal γ-globulin was then digested with papain. Although comparable amounts of *A* and *C* were obtained, the anti-Rh activity was limited to fraction *A*. When the 'basic' fraction of anti-Rh γ-globulin was mixed with normal γ-globulin, the anti-Rh activity was confined almost exclusively to papain fraction *C* of the resultant mixture.

These observations indicate that the differences in the net charge of γ-globulin molecules in the serum of a single individual are reflected in the varying proportions of *A* to *C*. The relative amounts of *A* and *C* depend on the charge of the parent γ-globulin. The fact that only these two fractions, *A* and *C*, are obtained from a parent γ-globulin consisting of molecules having a spectrum of charges is probably attributable to the chromatographic method[6,20]. Since molecular weight data[6,9,11] indicate

that each 7 *S* γ-globulin molecule consists of two sub-units of the *A*–*C* type and one of the *B* type, it seems likely that some γ-globulin molecules contain either two *A* or two *C* sub-units, that is, two identical univalent fragments. Present results, however, are not sufficient to support the hypothesis that this holds true for all γ-globulin molecules. The fact that the electrophoretically homogeneous myeloma proteins (Table 1) yielded either almost two parts of *A* and almost no *C*, or two parts of *C* and almost no *A* (relative to one part of *B*), is in keeping with this hypothesis. (The trace amounts of *A* in myelomas 1 and 4 and of *C* in myelomas 2 and 3 presumably are due to small amounts of normal (non-myeloma) γ-globulin in the myeloma sera.)

In parallel experiments, the 3·5 *S* fragments of two 7 *S* γ-globulins containing incomplete anti-Rh activity were prepared by sequential treatment with pepsin and a reducing agent[21]; their behaviour when tested by antiglobulin, ficin and albumin methods was identical with that of similar fragments prepared with papain. The 3·5 *S* fragments of a human 7 *S* anti-*A* isoagglutinin also failed to agglutinate the appropriate erythrocytes, whether or not the latter were treated with enzymes or suspended in a high protein medium. However, combination of these fragments with the homologous antigens did occur, as shown by the occurrence of agglutination when an antiserum specific for the 3·5 *S* fragments was added. (In a few experiments, the digest of the antibody appeared to retain a slight ability to agglutinate erythrocytes treated with enzymes or suspended in a high protein medium, but in each case this was found to be caused by the persistence of small amounts (1–3 per cent) of intact 7 *S* material, removable by gel-filtration on 'Sephadex *G*-200'.) Similar results have been obtained previously with rabbit bivalent 7 *S* agglutinating antibodies[18].

The results obtained with the papain digest of the incomplete anti-Rh antibodies merit further comment, in that they provide some basis for speculation on the relationship of the structure of such antibodies to their 'incomplete' character. The serological behaviour of the papain digest and its *A* and *C* fractions is what would be expected of truly univalent antibodies, that is, combination with antigen (as detected by the antiglobulin technique), but inability to cause agglutination of appropriate erythrocytes, even when the latter are pre-treated with ficin or suspended in a high protein medium. The parent 'incomplete' antibody, which reacts by all three methods, presumably is bivalent[18]. The means by which enzyme treatment of erythrocytes or the presence of a high protein medium enables 'incomplete' blood group antibodies to produce agglutination remain to be clarified. The mechanisms responsible for the serological behaviour of 'incomplete' antibodies are at present being investigated

in studies with univalent fragments of chromatographically sub-fractionated human γ-globulin.

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[1] Porter, R. R., *Nature*, **182**, 670 (1958).
[2] Porter, R. R., *Biochem. J.*, **73**, 119 (1959).
[3] Nisonoff, A., and Woernley, D. L., *Nature*, **183**, 1325 (1959).
[4] Karush, F., *Fed. Proc.*, **18**, 577 (1959).
[5] Nisonoff, A., Wissler, F. C., and Woernley, D. L., *Arch. Biochem. Biophys.*, **88**, 241 (1960).
[6] Palmer, J. L., Mandy, W. J., and Nisonoff, A., *Proc. U.S. Nat. Acad. Sci.*, **48**, 49 (1962).
[7] Stelos, P., Radzimski, G., and Pressman, D., *J. Immunol.*, **88**, 572 (1962).
[8] Putnam, F. W., Hsiao, S. H., and Tan, M., *Fed. Proc.*, **19**, 77 (1960).
[9] Franklin, E. C., *J. Clin. Invest.*, **39**, 1933 (1960).
[10] Hsiao, S. H., and Putnam, F. W., *J. Biol. Chem.*, **236**, 122 (1961).
[11] Deutsch, H. F., Stiehm, E. R., and Morton, J. I., *J. Biol. Chem.*, **236**, 2216 (1961).
[12] Edelman, G. M., Heremans, J. F., Heremans, M. T., and Kunkel, H. G., *J. Exp. Med.*, **112**, 203 (1960).
[13] Sober, H. A., Gutter, F. J., Wyckoff, M. M., and Peterson, E. A., *J. Amer. Chem. Soc.*, **78**, 756 (1956).
[14] Scheidegger, J. J., *Intern. Arch. Allergy*, **7**, 103 (1955).
[15] Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. S., *J. Biol. Chem.*, **193**, 265 (1951).
[16] Dunsford, I., and Bowley, C. C., *Techniques in Blood Grouping* (Charles C. Thomas, Springfield, 1956).
[17] Kunkel, H. G., in *Methods of Biochemical Analysis*, **1**, 141 (Interscience Publishers, New York, 1954).
[18] Fudenberg, H., Mandy, W. J., and Nisonoff, A., *J. Clin. Invest.*, **41**, 2123 (1962).
[19] Deutsch, H. F., Thorpe, N. O., and Fudenberg, H. H., *Immunol.* (in the press).
[20] Mandy, W. J., and Nisonoff, A., *J. Biol. Chem.*, **238**, 206 (1963).
[21] Nisonoff, A., Markus, G., and Wissler, F. C., *Nature*, **189**, 293 (1961).

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